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Mechanisms of Mitochondrial Damage in Keratinocytes by Pemphigus Vulgaris Antibodies*

Mina Kalantari-Dehaghi,[‡,1] Yumay Chen,[§,1] Wu Deng,[§] Alex Chernyavsky,[‡] Steve Marchenko,[‡] Ping H. Wang,[§¶,2] and Sergei A. Grando[‡¶,3]

From the Departments of [‡]Dermatology,
[§]Medicine, and
[¶]Biological Chemistry and
the Institute for Immunology, University of California, Irvine, California 92697
[2] To whom correspondence may be addressed: C240 Medical Sciences 1, University of California, Irvine, CA 92697., Tel.: Phone: 949-824-6981; Fax: 949-824-2200; E-mail: phwang@uci.edu.
[3] To whom correspondence may be addressed: 134 Sprague Hall, University of California, Irvine, CA 92697., Tel.: Phone: 949-824-2713; Fax: 949-824-2993; E-mail: sgrando@uci.edu.
[1]Both authors contributed equally to this work.

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Abstract

The development of nonhormonal treatment of pemphigus vulgaris (PV) has been hampered by a lack of clear understanding of the mechanisms leading to keratinocyte (KC) detachment and death in pemphigus. In this study, we sought to identify changes in the vital mitochondrial functions in KCs treated with the sera from PV patients and healthy donors. PV sera significantly increased proton leakage from KCs, suggesting that PV IgGs increase production of reactive oxygen species. Indeed, measurement of intracellular reactive oxygen species production showed a drastic increase of cell staining in response to treatment by PV sera, which was confirmed by FACS analysis. Exposure of KCs to PV sera also caused dramatic changes in the mitochondrial membrane potential detected with the JC-1 dye. These changes can trigger the mitochondria-mediated intrinsic apoptosis. Although sera from different PV patients elicited unique patterns of mitochondrial damage, the mitochondria-protecting drugs nicotinamide (also called niacinamide), minocycline, and cyclosporine A exhibited a uniform protective effect. Their therapeutic activity was validated in the passive transfer model of PV in neonatal BALB/c mice. The highest efficacy of mitochondrial protection of the combination of these drugs found in mitochondrial assay was consistent with the ability of the same drug combination to abolish acantholysis in mouse skin. These findings provide a theoretical background for clinical reports of the efficacy of mitochondria-protecting drugs in PV patients. Pharmacological protection of mitochondria and/or compensation of an altered mitochondrial function may therefore become a novel approach to development of personalized nonhormonal therapies of patients with this potentially lethal autoimmune blistering disease.

Keywords: Animal Models, Autoimmune Diseases, Mitochondria, Pemphigus, Skin

Introduction

Pemphigus vulgaris (PV)[4] is a lifelong, IgG autoantibody-mediated blistering disease affecting oral/esophageal surfaces and/or skin. Patients develop blisters and nonhealing erosions caused by suprabasal split within the stratified squamous epithelium. Prior to the introduction of therapy with oral corticosteroids in the 1950s, PV had a dismal natural course with a 50% mortality rate at 2 years and 100% mortality rate by 5 years after onset of the disease. Although systemic corticosteroid treatment is lifesaving, the high dose and prolonged courses required for disease control are associated with significant adverse effects, including death (1, 2). At present, mortality remains at a relatively high level of up to 12% (3). The optimal therapeutic strategy has not been established, the steroid-sparing drugs are associated with significant adverse effects themselves, and it is unknown which is the preferable steroid-sparing agent (4). The ultimate goal of pemphigus research is to develop an effective treatment modality that would allow patients to achieve and maintain clinical remission without the need for systemic corticosteroids.

The mechanism of detachment of keratinocytes (KCs) in PV, termed acantholysis, remains obscure and is a subject of intensive research. On the cell membrane of KCs, IgG autoantibodies produced by PV patients react with desmoglein (Dsg) 3 ± 1 and other self-antigens and elicit downstream signaling events causing cell shrinkage, detachment from neighboring KCs, and rounding up (reviewed in Ref. 5). Although the pathogenic role of anti-Dsg antibodies is well characterized (reviewed in Ref. 6), our recent studies using proteomic technology identified novel targets of PV autoimmunity (7, 8). Various anti-keratinocyte antibodies may concur to cause blistering by acting synergistically with anti-Dsg antibodies, as has been described through the “multiple hit” hypothesis (9). Our focus on mitochondrial targets of PV autoimmunity stems from classic and modern studies placing mitochondrial damage within the pathophysiological loop. The mitochondrial dysfunction in the lesional skin of PV patients had been suggested by an increase of lipid peroxidation and peroxidant-antioxidant balance, reflecting an increased production of reactive oxygen species (ROS) (10, 11) and measuring oxidative stress (12), respectively; abnormal activities of the mitochondrial enzymes oxidoreductase, adenosine triphosphatase, and $NADH_2$-cytochrome *c* reductase (13–15); and skewed balance between oxybiotic and anoxybiotic metabolism toward the latter (15).

Studies of mitochondrial antibodies (MtAbs) in pemphigus were pioneered by Geoghegan and Jordon in 1992 (16) and further developed by our group. We became interested in MtAbs because we sought to elucidate the mechanism of intrinsic apoptosis of KCs in PV originally demonstrated by us (17) and confirmed by others (18, 19). The direct evidence that MtAbs are critical to disease pathology, rather than a bystander phenomena in PV, was provided by the studies demonstrating that PV IgGs enter KCs and specifically bind to a number of mitochondrial proteins, which is associated with the mitochondrial damage manifested by cytochrome *c* release (20). Most importantly, adsorption of MtAbs abolished the ability of the IgG fraction of PV serum (PVIgG) to cause keratinocyte detachment (*i.e.* acantholysis) and skin blistering, thus illustrating their pathophysiological significance. Using a protein microarray approach, we have recently analyzed antigen specificities of autoantibodies of a large cohort of pemphigus patients and identified a number mitochondria-associated proteins targets by MtAbs (8). The most common targeted for MtAbs in PV are listed in Table 1. On the other hand, there is growing evidence that the pharmacological agents that can protect mitochondria, such as minocycline, nicotinamide (also called niacinamide), and cyclosporine A, are therapeutic in PV patients (see Table 2). Thus, taken together, the existing data suggest

strongly that PVIgG binding to KCs causes mitochondrial dysfunction and oxidative stress, triggering apoptosis of KCs and acantholysis (also called apoptolysis (21)), and that correction of mitochondrial function may be therapeutic in PV.

In this study, we employed assays of mitochondrial functions to identify changes in the vital mitochondria functions, such as O_2 respiration, mitochondrial membrane potential ($\Delta\Psi_m$), and intracellular production of ROS, in KCs treated with the sera from PV patients and healthy donors. The obtained results indicated that MtAbs produced by PV patients can disrupt the electron transfer chain, resulting in a loss of electrochemical gradient across the inner membrane, increase ROS production, and reduce the ability of KCs to respond to stress. Although the MtAbs of individual PV patients elicited unique patterns of mitochondrial damage, mitochondria-protecting drugs exhibited a uniform protective effect. Their therapeutic activity was validated in the passive transfer PV model in neonatal BALB/c mice. The obtained results explain the mechanism of therapeutic action of mitochondria-protecting drugs in PV patients and suggest novel avenues for treatment of this potentially lethal immunoblistering disease.

MATERIALS AND METHODS

Test Sera and Cells We tested six PV patient and six normal serum specimens. This study was approved by the University of California Irvine Human Subjects Review Committee. The diagnosis of PV was made based on the results of comprehensive clinical and histological examinations and immunological studies that included both direct immunofluorescence of skin biopsies and indirect immunofluorescence of the patients' sera on various epithelial substrates. The titer of “intercellular” antibodies determined on monkey esophagus ranged from 1/320 to 1/2560. The presence of anti-Dsg 1 and Dsg 3 antibodies in each PV serum was established using the MESACUP Dsg 1 and Dsg 3 ELISA test system (MBL, Nagoya, Japan). The index values for Dsg 1 antibodies ranged from 26 to 72, and those for Dsg 3 antibodies ranged from 65 to 164, *i.e.* were unequivocally positive. Patient specimens were deidentified prior to testing. As controls, we used normal human sera purchased from Bioreclamation, Inc. (Westbury, NY). The Het-1A cell line, an established clonal population of SV40-immortalized human esophageal squamous epithelial cells (*i.e.* KCs) widely used for the studies of apoptosis (22), was purchased from American Type Culture Collection (Manassas, VA; catalogue no. CRL-2692) and propagated in the Clonetics brand bronchial cell medium without retinoic acid (Cambrex Bio Sciences, Walkersville, MD), as detailed by us elsewhere (23).

Analysis of Mitochondrial O_2 Respiration by Extracellular Flux Measurement To measure mitochondrial function in Het-1A cells, we employed a Seahorse Bioscience XF24 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA) and followed the manufacturer's protocol. Briefly, KCs were plated in a 0.2% gelatin coated 24-well Seahorse XF-24 assay plate at 3×10^4 cells/well and grown for 16 h before being treated in quintuplicate with either PV patient's or normal human sera at a final concentration of 4% for another 24 h in the culture medium. On the day of metabolic flux analysis, the cells were washed once with freshly prepared Krebs-Henseleit buffer (111 mM NaCl, 4.7 mM KCl, 2 mM $MgSO_4$, 1.2 mM Na_2HPO_4, 2.5 mM glucose, and 0.5 mM carnitine, pH 7.4) and incubated in Krebs-Henseleit buffer at 37 °C in a non-CO_2 incubator for 1 h. Three base-line measurements of oxygen consumption rate (OCR) were taken before sequential injection of the following mitochondrial inhibitors and final concentration: oligomycin (1 μg/ml), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (3 μM), and rotenone (0.1 μM). Measurements were taken after addition of each of the three inhibitors. The OCR values were automatically calculated and recorded by the Seahorse XF-24 software. The basal respiration was calculated by averaging the three measurements of ORC before injection of inhibitors. The proton leak was

calculated using OCR measurement after oligomycin injection minus OCR measurement after rotenone injection.

Measurement of Mitochondrial $\Delta\Psi_m$ Using the JC-1 Dye The changes in $\Delta\Psi_m$ induced by test sera were measured using a standard protocol (24). Briefly, Het-1A cells were plated in a 6-well plate at a density of 5×10^4 per well, incubated for 16 h to allow cells to adhere to the dish surface, after which the cells there were either left untreated (negative control) or exposed for 24 h to PV or control sera at a final concentration of 4% of the total volume per well. Next, experimental and control cells were exposed to the fluorescent cationic dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbo-cyanine iodide) for 25 min before observation at green and red emission wavelengths using a Zeiss AxioPlan II fluorescence microscope (Carl Zeiss, Thornwood, NY). Intact mitochondrial membrane retains the JC-1 dye and forms J-aggregate (orange-red fluorescence), whereas depolarization of membrane decreases the ability to retain the dye that remains as a monomer (green fluorescence). Increased green fluorescence and deceased orange-red fluorescence thus indicated a loss of $\Delta\Psi_m$. In the absence of apoptosis, the lipophilic JC-1 dye bearing a delocalized positive charge accumulates in the mitochondrial matrix and stains the mitochondria bright red, whereas in mitochondrial apoptotic $\Delta\Psi_m$ dissipates, the cells display green fluorescence.

Intracellular ROS Measurement The measurement of intracellular ROS was performed in accordance with the manufacturer's protocol. The Het-1A cells were incubated and treated as described above in the $\Delta\Psi_m$ measurement section. Briefly, to measure intracellular ROS, the treatment medium was removed, and 5 μM c-H_2DCFDA-AM (Invitrogen) in PBS, pH 7, was added to each well and incubated for 15 min at 37 °C. The cells were then photographed using a fluorescence microscope. C-H_2DCFDA-AM is hydrolyzed by intracellular esterases and oxidized to fluorescent carboxy-DCF (excitation, 488 nm; emission, 525 nm).

FACS Measurements of $\Delta\Psi_m$ and ROS For the FACS analysis, the experimental and control Het-1A cells were incubated at 37 °C with either 5 μM of JC-1 dye for 60 min ($\Delta\Psi_m$ measurement) or 5 μM of c-H_2DCFDA-AM for 15 min (ROS measurement), washed three times with PBS, trypsinized, and resuspended in PBS. The fluorescence was measured with a BD LSR II flow cytometer (excitation, 488 nm; emission, 576 nm), and the data were analyzed using the BD FACSDiVa software.

Morphometric Assay of Acantholysis in Vivo PVIgGs were isolated by FPLC protein G affinity chromatography using the FPLC System purchased from Amersham Biosciences following the manufacturer's protocol, as detailed elsewhere (17). Minocycline, nicotinamide, and cyclosporine A were purchased from Sigma-Aldrich. One-day-old pups delivered by the BALB/c mice purchased from the Jackson Laboratory were used to investigate the effect of mitochondria-protecting drugs on the extent of epidermal acantholysis induced by passive transfer of 1 mg/g of body weight of PVIgG. The pups were injected subcutaneously with PVIgG with or without test drugs and examined 24 h later for the extent of epidermal acantholysis, as detailed elsewhere (20). Briefly, the euthanized animals were snap frozen in liquid nitrogen, cross-sectioned at the umbilicus level, embedded into the OCT compound (Miles Scientific, Naperville, IL), and stained by hematoxylin and eosin. Five random microscopic fields in each skin section were captured at magnification 10×, using a Macintosh computer attached to the Axiovert 135 inverted microscope. The images were printed, and the extent of acantholysis was computed directly on the prints by measuring the length of the areas in the epidermis in which suprabasal cell detachment spread along more than four adjacent basal cells.

Statistical Analysis The data were analyzed using analysis of variance against an α level of 0.05 and

presented as the mean ± S.D. The graphs were made using GraphPad Prism 5.

RESULTS

Antigen Specificities of MtAbs Determined in Our Proteomic Study Previously, we had demonstrated that PV patients develop autoantibodies that can find their way to mitochondria in KCs and react with a versatile group of mitochondrial proteins, which contributed to PV-like cell detachment in monolayers of human KCs and epidermis of neonatal mice (20). Most recently, we have tested 264 pemphigus and 138 normal control sera on the multiplexed protein array platform containing 701 human genes encompassing many keratinocyte molecules, including 283 mitochondria-associated proteins (8). A detailed analysis of the supplementary data published by us online (8) identified the most common antigens recognized by MtAbs and the percentage of PV patients and healthy people producing such MtAbs (Table 1). Based on the known functions of mitochondrial proteins most commonly targeted in PV, the following mitochondrial pathways might be subject to dysfunction: oxidative phosphorylation, O_2 respiration, and production/inactivation of ROS.

PV Sera Alter Mitochondrial O_2 Respiration in KCs To identify the direct effect of MtAbs on O_2 respiration, we performed a Seahorse Bioscience XF24–3 extracellular flux analysis of OCR in KCs treated with PV or normal human sera. Although none of four tested normal sera changed basal mitochondrial respiration, all PV sera significantly increased this mitochondrial function (Fig. 1, *A* and *B*). The PV sera also significantly increased proton leakage from treated cells (Fig. 1*C*). These results suggested that MtAbs produced by PV patients increase the ROS production in KCs and reduce the ability of these cells to respond to stress, which can trigger the mitochondria mediated (intrinsic) apoptosis.

PV Sera Alter $\Delta\Psi_m$ in KCs Exposure of KCs to PV, but not normal, sera caused dramatic changes in the mitochondrial membrane potential, manifested by disappearance of orange-red fluorescence of JC-1 dye with simultaneous increase in green fluorescence of cultured cells (Fig. 2*A*). A significant drop in the mitochondrial membrane potential in KCs treated with PV sera was confirmed by measurements of $\Delta\Psi_m$ using FACS (Fig. 2*B*). These results indicated that binding of MtAbs to mitochondrial target proteins disrupts the electron transfer chain, resulting in a loss of electrochemical gradient across the inner membrane.

PV Sera Increase Intracellular ROS Production in KCs When intracellular ROS production was measured by the c-H_2DCFDA-AM dye labeling of cultured KCs, we observed a drastic increase of cell staining in response to treatment by each individual PV serum, compared with normal sera (Fig. 3*A*). Similar results were obtained in FACS analysis (Fig. 3*B*), confirming that treatment with PV sera significantly increases the number of c-H_2DCFDA-AM-positive KCs, compared with normal controls. These results indicated that binding of MtAbs to keratinocyte mitochondria increases intracellular ROS production.

Mitochondria-protecting Drugs Abolish Deleterious Effects of PV Sera on Mitochondrial O_2 Respiration in KCs To evaluate direct effects of the mitochondria-protecting drugs minocycline, nicotinamide, and cyclosporine A, given alone or in combination, on the KCs exposed to PV serum, we measured OCR in real time in an Seahorse XF Efflux Analyzer and calculated proton leak. KCs exposed to different PV sera showed differential responses to test drugs (Fig. 4*A*). Drug combination regimen showed the highest efficacy in abolishing the PV serum-induced elevation of OCR and proton leak (Fig. 4*A*). These results indicated that although each PV patient's MtAbs display a unique pattern of mitochondrial damage, a combination of mitochondria-protecting drugs exhibits a uniform protective action.

Treatment of Experimental PV in Neonatal Mice by Pharmacologically Protecting the Mitochondria Next, we sought to confirm the therapeutic efficacy of the mitochondria-protecting drugs in the passive transfer model of PV in neonatal BALB/c mice. Coadministration of PVIgG and each of test drugs abolished the acantholytic activity of PVIgG, albeit with different efficacy (Fig. 4*B*). A decrease of PVIgG-induced epidermal splitting in mice treated with minocycline or cyclosporine A reached statistical significance ($p < 0.05$). Remarkably, the highest efficacy of mitochondrial protection of the combination of minocycline, nicotinamide, and cyclosporine A observed in the mitochondrial assay was consistent with the ability of this combination to almost completely abolish acantholysis in mouse skin (Fig. 4*B*). Thus, preventing the MtAb-dependent mitochondrial damage protected KCs from PVIgG-induced detachment and skin blistering.

DISCUSSION

PV is a complex, multifactorial disease (5, 25). The fact that adsorption of MtAbs from PVIgGs abolished the ability of PVIgGs to induce acantholysis (20) provided strong evidence of the indispensible role of mitochondrial damage in the disease mechanism. In this study, we demonstrated for the fist time dramatic changes in the mitochondria O_2 respiration, dissipation of $\Delta\Psi_m$, and drastic increases in the intracellular levels of ROS. These changes can induce intrinsic apoptosis, which is consistent with observations that PVIgG binding to KCs is associated with mitochondrial damage manifested by cytochrome *c* release and activation of caspase 9 (17–20). Because the mode of mitochondrial damage by MtAbs depends on the biological function of targeted protein, the exact primary mechanisms of mitochondrial damage apparently differ from patient to patient, in keeping with striking variations in the disease severity and response to treatment among different PV patients (26). The differential patterns of mitochondrial damage in PV have been also suggested by the fact that the mitochondrial function in KCs exposed to different PV sera showed differential responses to the same mitochondria-protecting drugs (Fig. 4*A*).

The structural-functional interrelationships of mitochondria-associated proteins targeted by MtAbs (Table 1) indicate that autoantibody binding to even a single mitochondrial target antigen can distort the neatly orchestrated cascade of interactions of enzymes and their substrates, producing a domino effect, resulting in the mitochondrial dysfunction. Because MtAbs produced by PV patients can bind their target proteins at both mitochondrial outer and inner membranes, as well as mitochondrial matrix, they can interfere with the biological processes taking place at these locations. Furthermore, because MtAbs recognized both mitochondrial- and nuclear-encoded proteins involved in the regulation or execution of specific mitochondrial functions (Table 1), the protein import into the mitochondria may also be altered, resulting in a pathologic remodeling of mitochondrial proteome.

The results of the present study clearly demonstrated that exposure of KCs to PV serum can disrupt their $\Delta\Psi_m$. This phenomenon can be explained based on the knowledge about involvement of mitochondrial structures in multiple physiological pathways, so that formation of an antigen-antibody complex in one location can influence several biochemical reactions. For example, binding of MtAbs to the complex I proteins, such as NADH dehydrogenase or electron transfer flavoprotein targeted in PV (Table 1), can interfere with electron exchange among the carriers and their access to succinate and NADH and affect their ability to establish a chemiosmotic gradient. Thus, disruption of $\Delta\Psi_m$ in KCs exposed to PV sera observed in this study can prevent electron transport (because of MtAb binding one or more electron carriers), which is an early event in apoptosis activated through the mitochondrial pathway (reviewed in Ref. 27).

It is well established that a delicate balance exists between the moderate ROS production to modulate physiological signaling and the overproduction of ROS that can lead to oxidative stress. ROS detoxification pathways exist to minimize oxidative damage. The presence of anti-superoxide dismutase MtAb in PV patients (Table 1) is in keeping with our observation that preincubation of KCs with PV sera led to excessive ROS production, thus precipitating changes in mitochondrial homeostasis. However, the mitochondria damaged by other types of MtAbs can also lead to ROS increase, creating a pathological process termed the "vicious cycle." For example, impairment in the mitochondrial electron transport that leads to a loss of $\Delta\Psi_m$ also can be responsible for an incomplete O_2 reduction, resulting in an increment in ROS production that further amplifies the generation of ROS. Such abnormalities can, in turn, trigger the cell death signaling cascade, wherein the executioner caspases cleave the cell adhesion molecules, such as desmosomal cadherins (reviewed in Ref. 28), leading to both cell detachment (acantholysis) and death (apoptosis), the unique form of keratinocyte demise in PV that we have tentatively termed apoptolysis (21). Further elucidation of the mitochondrial mechanisms of apoptolysis in PV should therefore improve our understanding of disease pathogenesis and facilitate development of personalized therapies based on the pharmacological correction of mitochondrial abnormalities in individual PV patients.

The results of the present study provide the first experimental evidence that pharmacological protection of mitochondrial function can prevent the skin blistering induced by PVIgG. These results shed light on the mechanism of therapeutic action of minocycline and nicotinamide, which have been empirically shown to be effective steroid-sparing agents in PV, as well as of the professional immunosuppressor cyclosporine A. Importantly, reports in the literature testify that in some patients PV can be controlled by the mitochondria-protecting drugs alone without the need for prednisone (29–35). Although these drugs belong to different chemical groups, each exhibiting unique pharmacological effects, they share the mitochondria-protecting activities (Table 2). However, except for empirically chosen combinations of minocycline and nicotinamide, no other combinations of the mitochondria-protecting drugs have been reported in PV. Our results demonstrated synergy among minocycline nicotinamide and cyclosporine A. The combination of drugs that most effectively protected mitochondrial respiration equally effectively antagonized the disease-causing activity of PVIgG in mouse skin. Thus, their synergistic potential is yet to be fully explored.

Because the mitochondria-protecting drugs used in this study are already in use in the treatment of PV patients, the obtained results translate into clinical practice. There is a strong rationale for the principally new treatment of patients with PV. The existing therapies do not allow one to reliably control acute PV without the systemic corticosteroid prednisone, which, although lifesaving, causes serious adverse events, including death, because of the need for its high dose (*e.g.*, 1–2 mg/kg/day) and prolonged (*e.g.*, 18 months) usage to achieve disease control (1, 2). Although the incidence of PV is only 1–16 per million population per year (36, 37), this disease represents a significant burden to health care professionals and the health care system because of the hazardous side effects of conventional immunosuppressive therapy requiring prolonged and frequent hospitalizations and high costs of modern treatment regimens (38). By identifying the dose-dependent effect, one should be able to increase drug efficacy, because the mitochondria-protecting drugs are being used in PV patients at their suboptimal doses. For example, whereas nicotinamide is given to PV patients at 1.5–2 g/day, this drug can be safely used at higher doses, like in patients with diabetes or schizophrenia (39). Likewise, cyclosporine A is usually used in PV at 0.5–1.5 mg/kg/day, which is much less compared with the dosages used in transplantology (40). These facts suggest that current suboptimal clinical activity of the mitochondria-protecting drugs used in PV can be improved by identifying their optimal combination through the laboratory screening of patient's serum

prior to treatment. Because the mitochondria-protecting drugs are expected to have different effects in different PV patients, as the mitochondrial pathways targeted by MtAbs are apparently unique to each patient, an individualized regimen will be needed to achieve the optimal therapeutic response.

In conclusion, the integrity of mitochondrial function is fundamental to cell life. Mitochondria are involved in many processes essential for cell survival, such as energy production, redox control, calcium homeostasis, and a number of metabolic and biosynthetic pathways. In addition, mitochondria often play an essential role in physiological cell death mechanisms (reviewed in Ref. 41). The results of the present study indicate that binding of PVIgG to mitochondrial targets can disrupt the electron transfer chain, resulting in a decline in ATP production, loss of electrochemical gradient across the inner membrane, and reduction in O_2 with increased generation of the ROS superoxide, hydrogen peroxide, and hydroxyl radical. These novel findings provide a theoretical background for clinical reports of the efficacy of mitochondria-protecting drugs in PV patients. Pharmacological protection of mitochondria and/or compensation of an altered mitochondrial function may therefore become a novel approach to the development of safer, *i.e.* nonhormonal, therapies of this severe autoimmune blistering disease.

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[4]The abbreviations used are:

PV pemphigus vulgaris
KC keratinocyte
ROS reactive oxygen species
Dsg desmoglein
MtAb mitochondrial antibody
PVIgG IgG fraction of PV serum
OCR oxygen consumption rate
c-H_2DCFDA-AM carboxy-2′,7′-dichlorodihydrofluorescein diactetate.

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Figures and Tables

TABLE 1

Mitochondrial proteins recognized by MtAbs from PV patients and healthy controls (8)

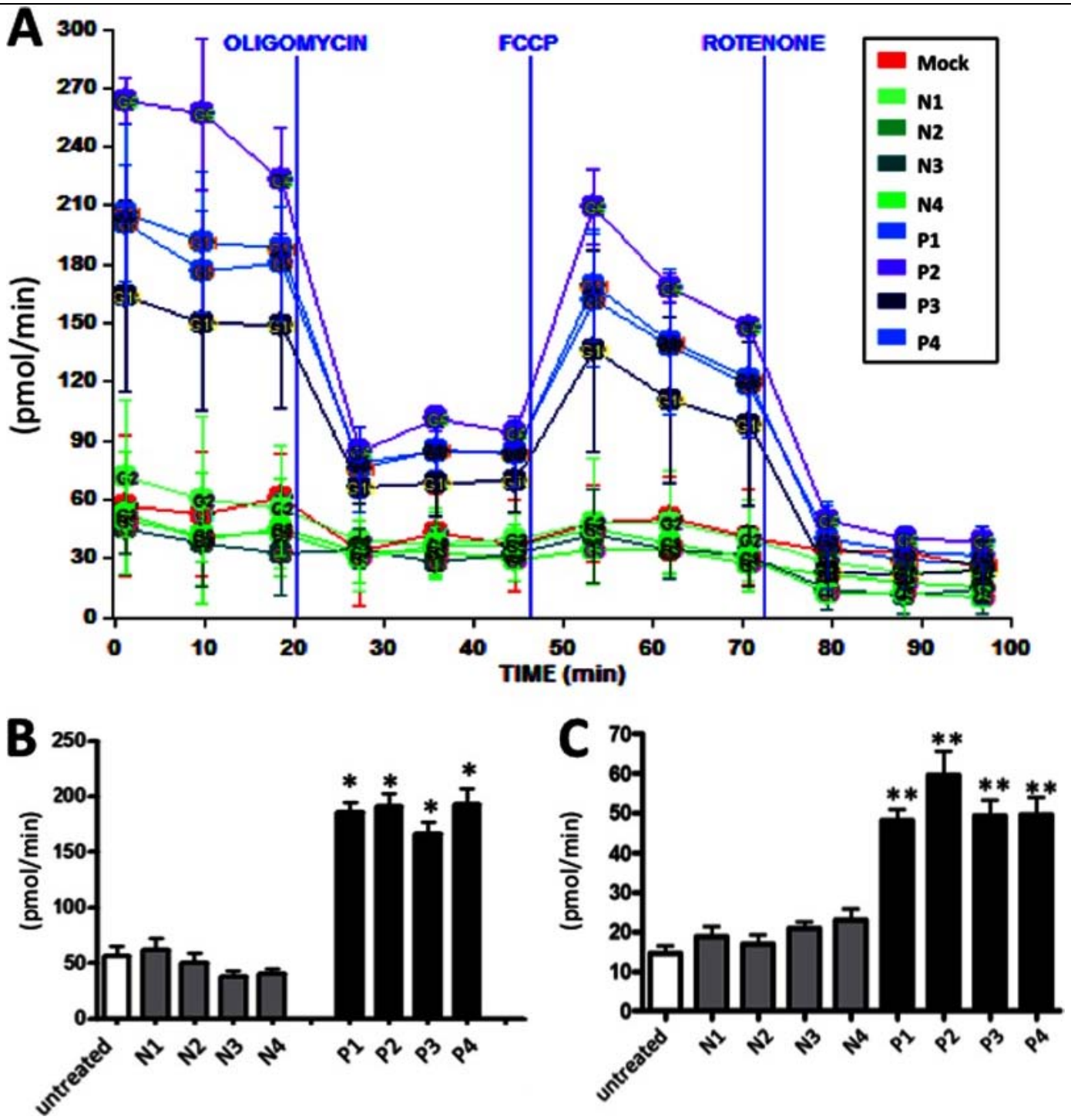
Gene name	Protein name	Localization	Biological process	Function	Frequency	
					PV	Control
					%	
PC	Pyruvate carboxylase	Matrix	Gluconeogenesis and lipid synthesis	Synthesis of phosphoenolpyruvate from pyruvate	32	5
PMPCB	Mitochondrial	Matrix	Mitochondrial	Cleaves presequences from	31	4

	processing peptidase β subunit		organization	mitochondrial protein precursors		
PDHA1	Pyruvate dehydrogenase E1 component α subunit, somatic form	Matrix	Glycolysis	Provides the primary link between glycolysis and TCA cycle by catalyzing the irreversible conversion of pyruvate into acetyl-CoA	30	3
FH	Fumarate hydratase (fumarase)	Mitochondrion	TCA cycle	Catalyzes the reversible hydration/dehydration of fumarate to malate	29	3
MLYCD	Malonyl-CoA decarboxylase	Mitochondrion	Regulation of TCA cycle	Catalyzes the conversion of malonyl-CoA into acetyl-CoA and carbon dioxide; without this enzyme, an excess of malonic acid inhibits TCA cycle	29	4
CRAT	Carnitine *O*-acetyltransferase	Inner membrane	Fatty acid and lipid metabolism	Specific for short chain fatty acids; may be involved in the transport of acetyl-CoA into mitochondria; acetylcholine synthesis	28	7
MAOB	Amine oxidase (flavin-containing) B	Outer membrane	TCA cycle	Catalyzes the oxidative deamination of biogenic and xenobiotic amines	27	5
PRODH	Proline oxidase	Matrix	Proline metabolism	Catalyzes the first step in proline degradation	25	6
FDXR-V2	NADPH:adrenodoxin oxidoreductase	Matrix	Electron transport; cholesterol, lipid, and steroid metabolism	The first electron transfer protein in all the mitochondrial P450 systems	25	6
NDUFA13	NADH dehydrogenase [ubiquinone] 1α subcomplex subunit 13; 16 kDa	Inner membrane	Electron transport; respiratory chain; apoptosis	A subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (complex I); involved in the interferon/all-*trans*-retinoic acid-induced cell death	24	6
PDK4	Pyruvate dehydrogenase kinase, isozyme 4	Matrix	Carbohydrate and glucose metabolism	Inhibits the mitochondrial pyruvate dehydrogenase complex, thus contributing to the regulation of glucose metabolism	24	4
ME3	NADP-dependent malic enzyme,	Matrix	Links the glycolytic and	Generates NADPH for oxidative decarboxylation of malate to	23	8

	mitochondrial		TCA cycles	pyruvate		
SOD2	Superoxide dismutase [Mn]	Matrix	Antioxidant defense	Catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide	23	2
ALDH4A1	Aldehyde dehydrogenase 4 family, member A1	Matrix	Links the urea and TCA cycles	Converts Δ1-pyrroline-5-carboxylate to glutamate	23	5
NDUFS6	NADH dehydrogenase [ubiquinone] iron-sulfur protein 6; 13 kDa	Inner membrane	Electron transport; respiratory chain	A subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (complex I)	22	5
ETFB	Electron-transfer-flavoprotein, β protein	Matrix	Electron transport	Transfers electrons to mitochondrial respiratory chain via ETF-ubiquinone oxidoreductase	21	3
NDUFA9	NADH dehydrogenase [ubiquinone] 1α subcomplex subunit 9; 39 kDa	Matrix	Electron transport; respiratory chain	A subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (complex I)	20	3
TIMM44	Mitochondrial import inner membrane translocase subunit	Inner membrane	Protein transport	Translocation of transit peptide-containing proteins from the inner membrane into the mitochondrial matrix in an ATP-dependent manner	20	4
ETFA	Electron transfer flavoprotein, α protein	Matrix	Electron transport	Transfers electrons to mitochondrial respiratory chain via ETF-ubiquinone oxidoreductase	19	4
CYB5B	Cytochrome b_5 type B; 21 kDa	Outer membrane	Electron transport	An electron carrier for several membrane bound oxygenases	19	1
ABAT-V1	4-Aminobutyrate aminotransferase, mitochondrial; 50 kDa	Matrix	Neurotransmitter degradation	Catalyzes the conversion of γ-aminobutyrate and L-β-aminoisobutyrate to succinate semialdehyde and methylmalonate semialdehyde, respectively	19	4
NDUFV3	NADH dehydrogenase [ubiquinone] flavoprotein 3; 9 kDa	Inner membrane	Electron transport; respiratory chain	A subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (complex I)	19	4
CPT1B	Crnitine *O*-	Outer	Fatty acid and	A mitochondrial transferase	18	5

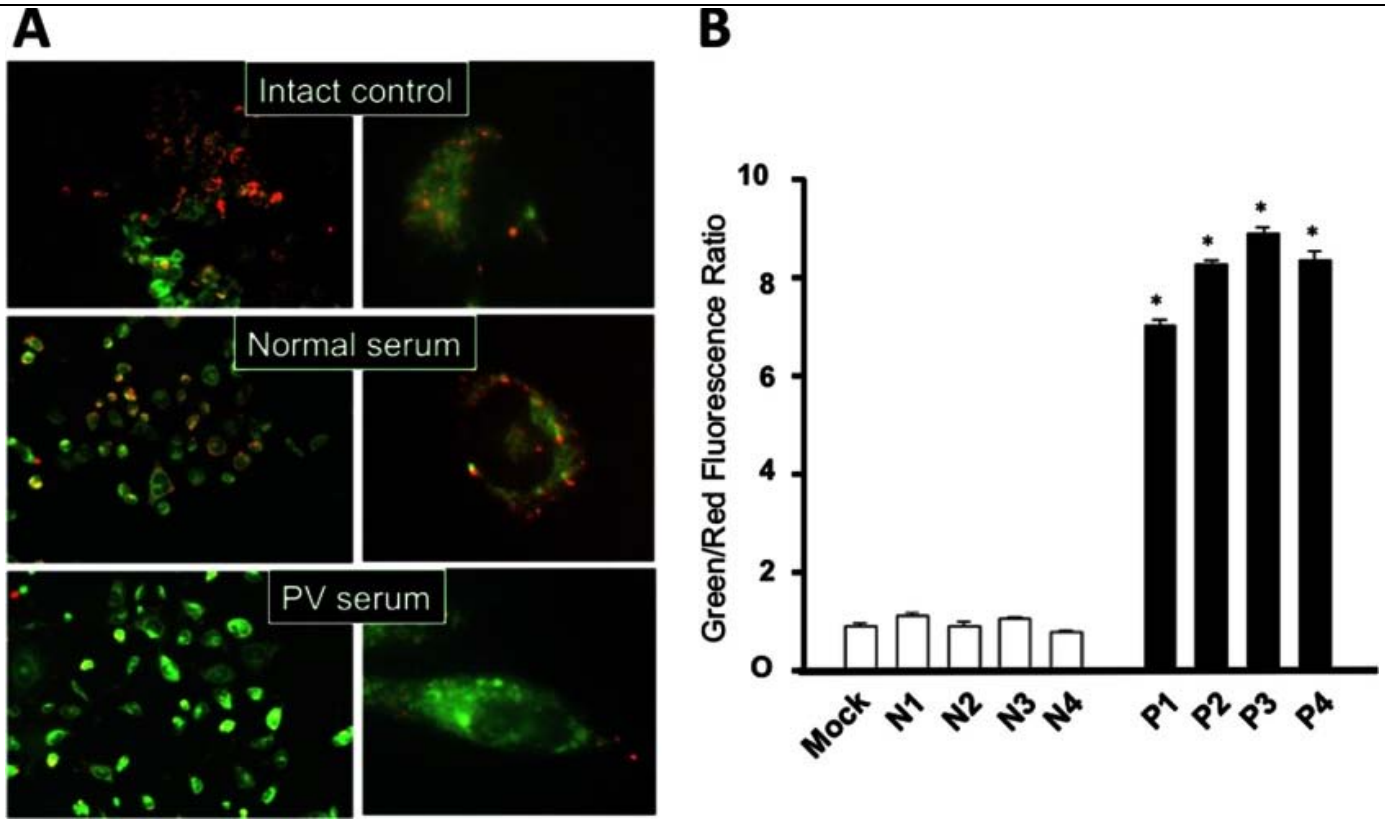
	palmitoyltransferase 1B	membrane	lipid metabolism	enzyme involved in the metabolism of palmitoylcarnitine into palmitoyl-CoA		
ME2	NAD-dependent malic enzyme	Matrix	TCA cycle	Catalyses the oxidative decarboxylation of malate to form pyruvate	18	6
NDUFB10	NADH dehydrogenase [ubiquinone] 1β subcomplex subunit 10	Matrix	Electron transport; respiratory chain	Accessory subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (complex I)	17	2

FIGURE 1.



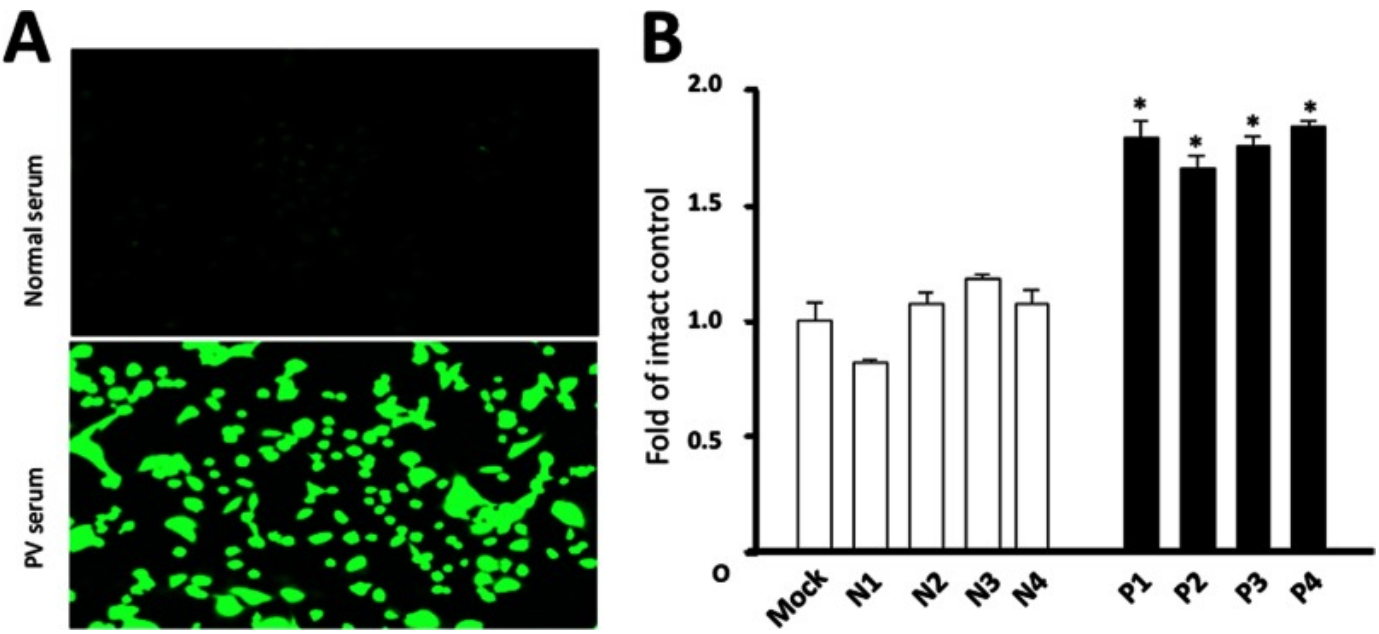
Deleterious effects of PV sera on mitochondrial O_2 respiration in KCs. *A*, time course of measurements of OCR in a Seahorse XF bioenergetics assay in Het-1A cells treated with four normal (N1–N4) or PV (P1–P4) sera at a final concentration of 4% *versus* no sera (mock), as detailed under "Materials and Methods." The data represent the means ± S.D. of quintuplicate measurements for each individual serum. *FCCP*, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. *B*, mean values of basal respiration calculated from three measurements before injection of oligomycin. *C*, the proton leak calculated using the OCR measurement after oligomycin injection minus the OCR measurement after rotenone injection. *, $p < 0.0001$; **, $p < 0.0002$ compared with mock treatment.

FIGURE 2.



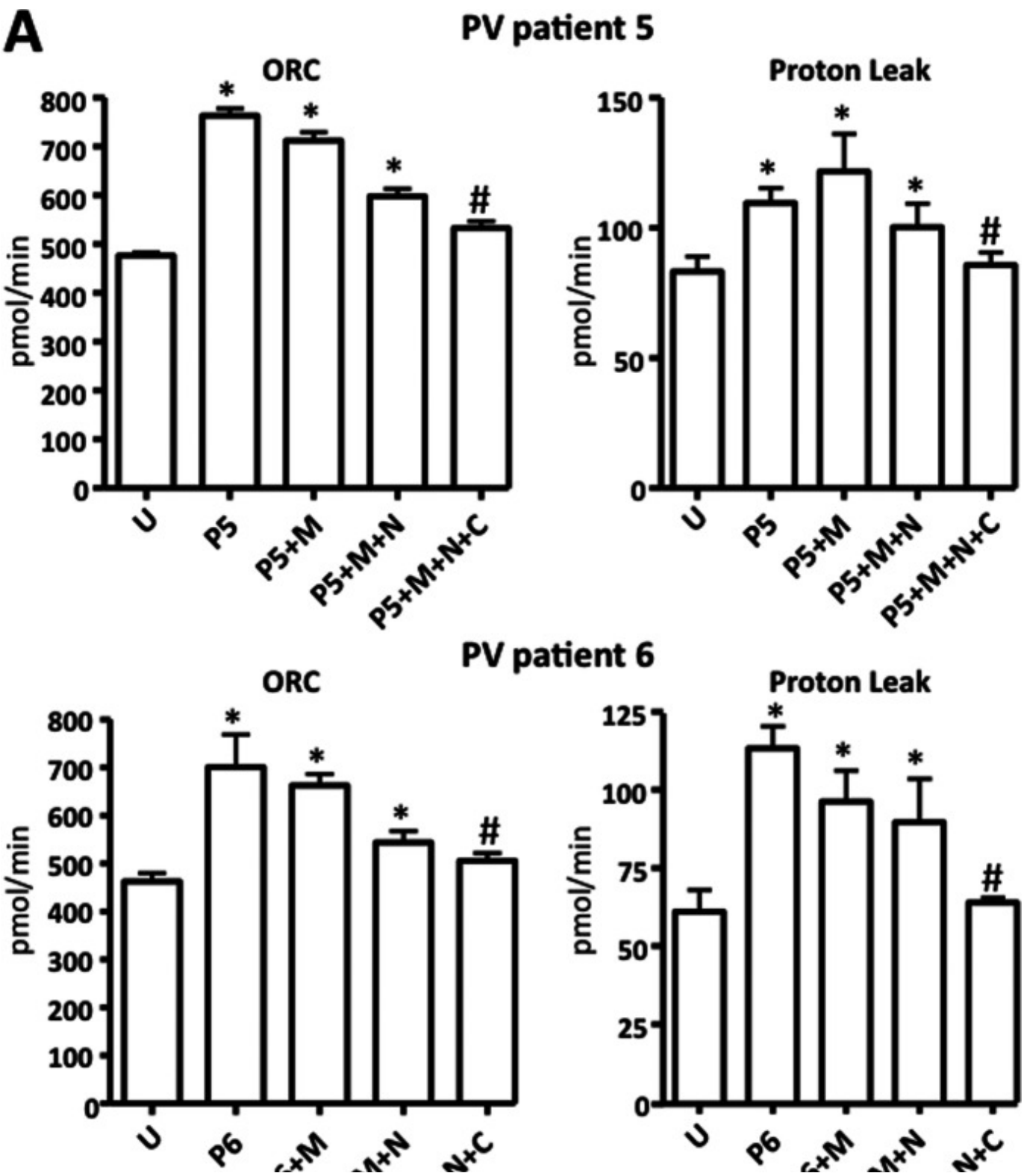
Effects of PV sera on mitochondrial membrane potential ($\Delta\Psi_m$) in KCs. *A*, representative images of the cultured Het-A1 cells treated with PV *versus* normal sera or left untreated (mock) and subjected to evaluation of JC-1 dye staining via fluorescence microscopy, as described under "Materials and Methods." The *right column* displays enlarged images of individual cells from the corresponding low power images of the *left column*. Loss of *red* fluorescence in cells treated with PV serum indicates loss of $\Delta\Psi_m$. *B*, FACS measurements of $\Delta\Psi_m$ in the Het-1A cells labeled with JC-1 dye. The ratio between *green* and *red* fluorescence for mock treated cells was set as 1. *, $p < 0.0001$ compared with mock treatment.

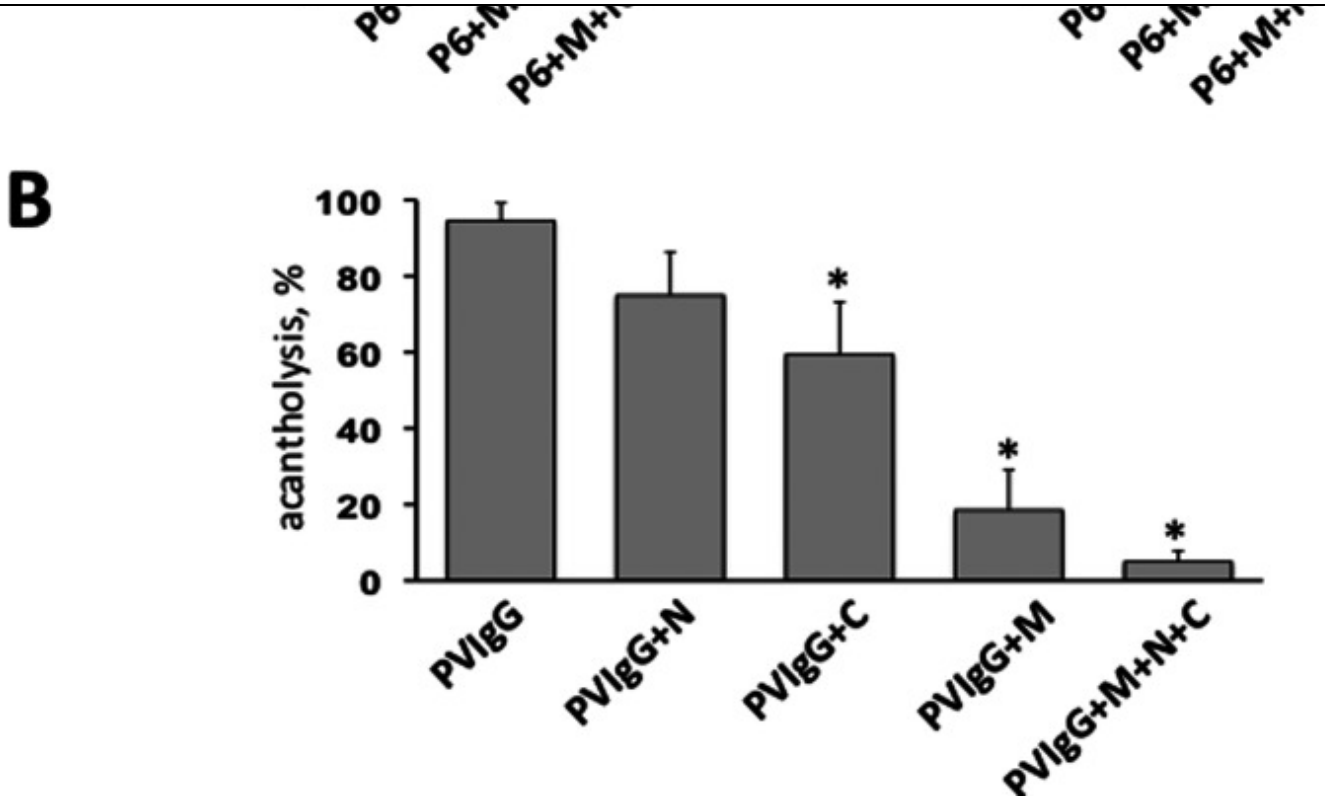
FIGURE 3.



Effects of PV sera on ROS production in KCs. *A*, representative images of cultured Het-A1 cells treated with PV *versus* normal sera and subjected to microscopic evaluation of the c-H_2DCFDA-AM dye staining via fluorescence microscopy, as described under "Materials and Methods." The images were taken with a Zeiss AxioPlan II fluorescence microscope using the same exposure time (40 ms). The increased intensity of cell fluorescence caused by treatment with PV serum indicates elevated ROS production. *B*, FACS analysis of intracellular ROS levels in Het-1A cells labeled with c-H_2DCFDA-AM dye. The fluorescence intensity of intact cells (mock) was set as 1. *, $p < 0.0001$ compared with mock.

FIGURE 4.





Mitochondria-protecting drugs abolish effects of MtAbs. *A*, differential effects of mitochondria-protecting drugs against the deleterious effects of PV from different patients. Representative results showing the ability of selective drugs to abolish an increase of OCR and proton leak. Het-1A cells were analyzed in real time with a Seahorse XF analyzer, as detailed under "Materials and Methods." The cells were pretreated with 0.05 mM minocycline (*M*) ± 10 mM nicotinamide (*N*) ± 0.1 μM cyclosporine A (*C*) for 30 min prior to incubation with sera from two PV patients. The untreated KCs (*U*) served as controls. The drugs had no effect on control cells (not shown). *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with PV sera without drugs. *B*, treatment of experimental PV in neonatal mice by protecting the mitochondria. Neonatal BALB/c mice ($n = 3$) received a single subcutaneous injection of 1 mg/g of body weight of PVIgG alone or in combination with test drug(s), and the extent of acantholysis in mouse epidermis was measured 24 h after injection using a morphometric assays described under "Materials and Methods." *, $p < 0.05$ compared with PVIgG injected alone.

TABLE 2

Drugs affecting mitochondrial stability and functions used to treat PV patients

Drug	Mechanism of protective action on mitochondria	Treatment of PV patients
Cyclosporine A	Inhibits mitochondrial permeability transition pore by preventing the binding of cyclophilin D to pore proteins (42, 43)	Used to treat PV patients as an immunosuppressor drug both systemically and topically; has been effective as a monotherapy in a few PV patients, but most commonly used as a steroid-sparing drug, inducing a prolong remission (29–31, 44–47); partially inhibited signs of experimental PV in mice (48)
Nicotinamide,	Nicotinamide is a precursor of the coenzyme NAD^+ used to	Used to treat PV patients both

also called niacinamide, an amide of nicotinic acid; one of two principal forms of the B-complex vitamin, B_3	generate ATP in the mitochondrial electron transport chain; can increase levels of NAD^+, NADH, $NADP^+$, and NADPH, inhibit poly(ADP-ribose) polymerase, and prevent/reverse the depolarization of the mitochondrial membrane (49); also can facilitate the anti-apoptotic nicotinergic signaling (50) by increasing the levels of endogenous cholinomimetic acetylcholine and the α7 nicotinic acetylcholine receptor agonist choline (51–53) and inhibition of the acetylcholine hydrolyzing enzyme acetylcholinesterase (54, 55)	systemically and topically (35), producing steroid-sparing effects; usually combined with either tetracycline (32–34, 56) or minocycline (57)
Minocycline	Inhibits mitochondrial permeability transition pore by reducing mitochondrial Ca^{2+} uptake, decreases release of mitochondrial pro-apoptotic, increases anti-apoptotic factors, and also modulates the activity of poly(ADP-ribose) polymerase (41)	Used a steroid-sparing drug to treat PV patients alone or in combination with nicotinamide (34, 57–60)